Combined use of borneol or menthol with labrasol promotes penetration of baicalin through rabbit cornea in vitro

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Abstract: The permeability of most drugs through the eyes is very limited, so finding safe and effective penetration enhancers is of high importance in current ophthalmology research. In this paper, we use a new approach that integrates Chinese and Western medicine to improve the corneal permeability of baicalin, a water- and fat-insoluble target drug, in vitro. Rabbits were divided into three groups. The first group was dosed with borneol (0.05%, 0.1%), menthol (0.1%, 0.2%), or Labrasol (1%, 2%) individually, the second was dosed with a combination of Labrasol with either borneol or menthol, and the third group received a control treatment. Compared with the control treatment, borneol, menthol, or Labrasol alone clearly improved the permeability of baicalin in vitro. Furthermore, the penetrating effects were significantly increased by combining the application of Labrasol with menthol or borneol. Among the various combined penetration enhancers, 0.1% borneol with 2% Labrasol achieved the best apparent permeability, approximately 16.35 times that of the control. Additionally, the calculation of corneal hydration level and the Draize test demonstrated the safety of these penetration enhancers to the rabbit corneas in vivo. This study confirms that the combined use of borneol or menthol, compounds both derived from Chinese herbs, with Labrasol can improve the corneal permeability of water- and fat-insoluble drugs.

Keywords: Penetration enhancer; borneol; menthol; labrasol.

INTRODUCTION

Currently, ophthalmic drugs are primarily administered either systemically or topically. Systemic administration requires a larger dose of drug to achieve the effective concentration in the eye, increasing the potential side effects within the whole body. Compared to systemic administration, local ocular application of drug requires a smaller dose since the drug can be delivered directly to the site of action. However, there can be additional complications that may be difficult for patients to accept (Delyfer et al., 2007). Intracconjunctival application, such as eye drops and ointments is the main route of ophthalmic drug administration. However, due to the type of barrier formed by the corneal structure, access of the drug to the eye is limited (Loftsson et al., 2008). This lack of permeability has become a major concern in the development of new ophthalmic drugs.

Traditional Chinese herbs have been used to treat disease, including those of the eye, for thousands of years (Cheng, 2000). Today the active components of these herbs are extracted and purified using modern pharmacological techniques. Although Chinese herbal medicines are used more often in Asian countries such as China and Japan (Nagaki et al., 2003), countries in Europe and North America are now beginning to actively study drugs containing individual components of these Chinese medicines (Bae et al., 2012). A number of these Chinese herbs are known to promote drug permeation through biological barriers, such as the cornea. In this study we concentrate on two Chinese herb derived compounds known to promote drug permeability, borneol and menthol whose chemical structures are shown in fig. 1. In addition we look at a common permeability enhancing compound which is not derived from Chinese herbs-Labrasol. Labrasol is the excipient for many drugs, and it is widely used in oral medications and topical ointments.

Our research group confirmed in a series of studies that the use of borneol or menthol, alone or in combination, can promote the penetration of drugs into the eye (Xu et al., 2011; Liu et al., 2012). At the same time, we found that the promoting effects of the two compounds on drug penetration are related to the water- and fat-solubility of the drug (Yang et al., 2009). Other researchers have shown that the compound Labrasol promotes the corneal permeability of drugs (Liu et al., 2009). In order to further examine the permeability enhancing effects of these drugs, we carried out a preliminary study using combinations of Labrasol with borneol or menthol. This study indicated that these combinations can change the water- or fat-solubility properties of drugs to enhance corneal penetration. In this study our main aim is to integrate the use of compounds derived from Chinese medicine (in this case, borneol and menthol) with those synthesized by modern pharmacological techniques (Labrasol).

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MATERIALS AND METHODS

Materials

Animals
Seventy-two New Zealand white rabbits (males and females, 2.5 to 3.0 kg) were supplied by the Animal Center of Harbin Medical University. Slit-lamp examinations were performed to exclude eye diseases. The animals were given a standard diet with free access to drinking water, and were housed at 25±1°C with a relative humidity of 50±5% and 12 hours of illumination daily. This study was approved by the Ethics Committee of Harbin Medical University.

Fig. 1: Chemical structures of the two Chinese herb derived compound as penetrate enhance
A: Menthol B: Borneol

Twenty-four rabbits were used for determination of the maximum safe drug concentration and optimal penetration enhancing concentration in vitro. Eight rabbits were used for determination of safe in vivo drug concentrations using the Draize test. The other forty rabbits (cornea: n=80) were randomly classified into two groups for in vitro experiments: control group (n=10) and study group (n=70). In the study group, the corneas were treated with borneol (0.05%, 0.1%), menthol (0.1%, 0.2%), or Labrasol (1%, 2%) individually (i.e. as a single penetration enhancer), as well as being treated with various combinations of Labrasol/borneol/menthol as shown in table 1.

Methods

Baicalin determination
The samples were detected by HPLC, with measurements taken at the wavelength of 280 nm. The mobile phase is acetonitrile-water (75: 25). Standards were used for the preparation of different gradients of baicalin and the standard curve was plotted. The linear range was 0.01 mg/L to 50mg/L.

Cornea preparation
White rabbits were euthanized by injecting 20mL air via the ear margin vein. The eye was enucleated and a circular incision was made in the sclera, 5 mm from the edge of the cornea. The iris, ciliary body, and lens were removed, and the scleral ring was carefully trimmed to a width of 2mm. The sample was rinsed three times with GBR solution and placed in GBR solution at 35°C for subsequent use. During the sample preparation process, care was taken to avoid contact with the corneal surface so as not to cause it any damage. The in vitro diffusion test was started within 20 minutes of sacrificing the animals.

Draize test in vivo
The Draize test (Draize et al., 1944) was used to assess eye irritation, using the highest of the concentrations to be used in the permeability test. Eight white rabbits were divided into two groups. One group was treated with baicalin-menthol-Labrasol and the other with baicalin-borneol-Labrasol. An eye examination was performed 24 hours before the test to exclude eye diseases. The test solution was dripped into the left conjunctival sac, and buffer (pH 7.4) was dripped into the right conjunctival sac as a control. The eyes were examined using a slit lamp at 1, 2, 4, 12, 24 and 48 hours after drug application, and the degree of the irritation of the cornea, conjunctiva, and the anterior chamber were evaluated for any sign of a toxic reaction. The eye irritation score (Draize et al., 1944) was given based on the degree of corneal opacity, iris irritation, conjunctival hyperemia, edema and secretions. The sum of the average scores for each was used to assess toxic reactions: 0 to 3.9 was considered no irritation; 4.0 to 8.9, mild irritation; 9.0 to 12.9, moderate irritation; and 13 to 16, severe irritation. All observations were made by the same individual.

Corneal hydration level
After each test, the hydration level of the tested cornea was determined. To determine Wₜ (wet weight) the cornea was simply dissected out and weighed. To determine W₈ (dry weight) the cornea was weighed after being dried in an oven at 60°C for 12 hours. The hydration level (HL) was calculated as HL = (1 - W₈/Wₜ) × 100%.
In vitro penetration enhancement study

The freshly prepared cornea sample was spread carefully between the donor chamber and receptor chamber of a Franz diffusion cell. The endothelial layer of the cornea faced the donor chamber. Preheated GBR solution (35°C) was added to the donor and receptor chambers and air bubbles in the receptor chamber were discharged. After allowing 15 minutes for equilibration, the GBR solution in the donor chamber was replaced with 2mL Ringer's (GBR) solution as the target solution. The sampled was stirred using a star-shaped stirring bar, and the device was placed in a constant temperature magnetic stirrer, with the temperature of the water bath held at 35±1°C. At the same time, the donor chamber and receptor chamber were sealed with a lid to prevent evaporation. A mixture of 95% O₂/5% CO₂ was pumped into the device to maintain oxygen concentration in the solution. Samples (200µL) were collected from the receptor chamber at 30, 45, 60, 90, 120, 150, 180, 210 and 240 minutes after starting the test, with replacement by an equal volume of GBR solution at each interval. The experiment lasted for 4 hours, and each test was repeated 5 times. The samples were stored at -20°C for subsequent use.

Data Processing

The cumulative osmolality (Q) was calculated using Formula 1, where \( C_n \) represents the concentration of the sample at time \( t \); \( C_i \) is the sample concentration at the time point before time point \( t \); \( V_0 \) is the volume of the receptor chamber; and \( V \) is the sample volume (0.2mL).

\[
Q_n = V_0(C_n + \sum_{i=1}^{n-1} C_i) - V_0C_0 + \sum_{i=1}^{n} C_i \quad \text{(Formula 1)}
\]

The apparent permeability (\( P_{\text{app}} \)) was calculated using Formula 2, where \( \Delta Q/\Delta t \) is the slope of the cumulative drug concentration Q and the initial straight line part of the tt curve; A is the effective diffusion area (0.5cm²) and \( C_0 \) is the initial concentration of the donor chamber. The number 3600 is the number of seconds converted from the number of hours.

\[
P_{\text{app}} = \Delta Q/(\Delta t \times C_0 \times A \times 3600)
\]

The enhancement ratios (ER) were calculated using Formula 3, where \( P_{\text{enh}} \) and \( P_{\text{ctrl}} \) represent \( P_{\text{app}} \) with and without penetration enhancer, respectively.

\[
ER = P_{\text{enh}}/P_{\text{ctrl}}
\]

STATISTICAL ANALYSIS

The data were analyzed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). All quantitative data were shown as mean ± standard deviation. The experimental data were analyzed using one-way ANOVA, and values of \( P<0.05 \) were considered statistically significant.

RESULTS

Draize test

In this study, we conducted the Draize test using two groups of drugs. Their components are shown in table 2. The concentrations used for the Draize test represented the maximum drug concentrations used in this study. The scores of Group 1 and Group 2 were 2.3±0.5 and 2.5±0.6, respectively. No obvious corneal or iris irritation symptoms occurred in either group. Conjunctival irritation, such as conjunctival hyperemia, disappeared within 24 hours of drug application, indicating that our experimental drugs are safe for the rabbit cornea.

Maximum safe concentration and optimal penetration enhancing concentration

Based on the method described in 2.2.4 and 2.2.5, the maximum safe concentrations of Labrasol, borneol and menthol were as follows: 3%, 0.4% and 0.2%. The optimal concentrations of Labrasol, borneol, and menthol when applied alone were found to be 2%, 0.2%, 0.1% respectively (table 3). These concentrations were then used as starting points to determine the optimal concentrations of each compound when used in combination with the other penetration enhancers.

Effects of combined use of enhancers

Based on the results for individual optimal concentrations, we combined, 0.1% and 0.2% menthol or 0.05% and 0.1% borneol with 1% or 2% Labrasol, respectively. Fig. 2 and 3 show plots of cumulative concentration versus time for the various combinations. The accumulate concentration of baicalin through cornea increased by time with the help of any penetration enhancers. However, combination of different concentrations of menthol or borneol with or without Labrasol is significant higher than any of single enhancer application. The specific penetration enhancers used, the steady state equation, \( P_{\text{app}} \) and enhancement ratios (ER) are listed in table 4.
Combined use of borneol or menthol with labrasol promotes penetration of baicalin

As expected, the results reveal that $P_{\text{app}}$ values for the penetration-enhancer groups were increased compared with that of the control group. More importantly, in the groups with combinations of penetration-enhancers, we found that the $P_{\text{app}}$ values were statistically increased compared to that of the same concentrations of menthol, borneol and Labrasol when used individually. For example, the $P_{\text{app}}$ values of 0.1% menthol used alone was increased from $4.16 \pm 0.06 \times 10^{-6} \text{ cm/s}$ to $6.16 \pm 0.10 \times 10^{-6} \text{ cm/s}$ when combined with 1% Labrasol, and to $17.61 \pm 0.27 \times 10^{-6} \text{ cm/s}$ when combined with 2% Labrasol. Similar results can be seen for the other compound combinations. The best $P_{\text{app}}$ values were achieved by the compound use of 2% Labrasol with 0.2% menthol ($P_{\text{app}}$: 17.61 $\pm 0.27 \times 10^{-6} \text{ cm/s}$) or 0.1% borneol ($P_{\text{app}}$: 19.95$\pm 0.20 \times 10^{-6} \text{ cm/s}$) (fig. 4).

DISCUSSION

In this study we were able to show the penetration-enhancing effects of borneol and menthol (Chinese medicine derived compounds) on the model drug baicalin. We also demonstrated that the penetration-enhancing effects of borneol and menthol were increased when used in combination with Labrasol- a well-known penetration enhancing compound not found in traditional Chinese medicine. Shizhen Li, one of the greatest Chinese herbalists in the Chinese Ming dynasty pointed out in his "Compendium of Materia Medica" that borneol can "open various orifices". This ancient observation is supported by modern research showing that borneol can safely promote the absorption of drugs through the skin and mucous membranes (Wan, 2012). In fact, the Chinese medicine known as Danshen Dripping Pill, has borneol as one of its main components, and has successfully passed phase II clinical trials supervised by the U.S. Food and Drug Administration (FDA). Menthol has been used medicinally in Japan for more than 2000 years and in modern Europe and the United States it is often used in the form of paste and cream (e.g. Tiger Balm and IcyHot). Many researchers have also reported the use of menthol as a penetration enhancer (Gao and Singh, 1988).

Labrasol, also known as caprylocaproyl macrogol glycerides, is a pharmaceutical excipient included in United States Pharmacopeia and the National Formulary, as well as the European Pharmacopoeia. It is not known to derive from any Chinese medicine. Labrasol has a large solubilization capacity, and it can improve the water- and fat-solubility of drugs. Many studies have shown that it has a penetration-enhancing role in the skin and intestinal mucosa (Bejugam, 2009; Rama et al., 2003). It is also reported that low concentrations of Labrasol promote corneal permeability (Liu et al., 2009). In this study we have demonstrated that combining these compounds is more effective for penetration enhancement in the cornea than using any of them individually.

Model drug safety

Two assays are commonly used to evaluate the safety of drugs used in the conjunctival sac. Firstly, in vitro corneal hydration levels can be used to assess corneal injury. Normal hydration levels range from 76% to 80%, with values higher than 83% indicating corneal injury. The Draize test may also be used to evaluate the safety of drugs used in the conjunctival sac (Draize et al., 1944). Here we used both of these assays. Using corneal hydration in vitro we were able to determine the maximum safe concentration of each compound when used as a penetration enhancer for baicalin at 200mg/L (table 2). We then added borneol (1%) or menthol (2%) to the solution containing baicalin and Labrasol (2%) and performed the Draize test in vivo using these different formulations. The results confirmed that under both in vitro and in vivo conditions, the drug concentrations used in our study were safe in rabbit eyes. The concentrations we determined to be safe are similar to those reported in the literature (Liu et al., 2009; Liu et al., 2012; Xu et al., 2011; Yang et al., 2009).

Model drug selection

Many of the active ingredients of traditional Chinese medicines are water- and fat-insoluble, limiting their applications in the medical field. In our study we used baicalin as our model drug because it is a well-known active ingredient in Chinese medicine and our intent was to find a method applicable to a wide variety of Chinese medicinal compounds in the field of ophthalmology. Baicalin is a flavonoid compound with antibacterial and antioxidant activities and is the main active ingredient of Scutellaria (Nagaki et al., 2003). It is currently used in ophthalmology to prevent the development of cataracts (Nagaki et al., 2003). The ability of baicalin to penetrate through the cornea by itself is lower than that of other model drugs, and so presents an excellent challenge for

Fig. 4: $P_{\text{app}}$ values in various groups with single or combined use of penetration enhancers.
penetration studies. Baicalin is water- and fat-insoluble with a higher molecular weight (446.37) compared with other model drugs. In a previous study Liu used Labrasol alone as a penetration enhancer for baicalin in vivo and found that it promoted the penetration of baicalin into the eye (Liu et al., 2009). Here we confirm these previous results, and expand them on by using Labrasol in combination with borneol and menthol.

**Penetration enhancing effects and mechanisms**

The main barriers to corneal drug penetration are the epithelial layer, which is difficult for water-soluble drugs to traverse, and the matrix layer, which is difficult for fat-soluble drugs to pass through. It is generally believed that neutral water-soluble substances of small molecular weight infiltrate through the Para cellular pathway, and neutral highly fat-soluble substances of small molecular weight infiltrate through the trans cellular pathway (Marnon et al., 2009). In a previous study, we found that the permeability of water-soluble drugs decreases as water solubility increases (Yang et al., 2009). That is, the more water-soluble a drug is, the less able to permeate the cornea. The results of the control group showed that the rate of baicalin alone passing through the cornea was low. However, in the presence of the various penetration enhancers the rate increased, as indicated by an increase in \( P_{\text{app}} \) values. The optimal effects of each penetration enhancer are different, which may be due to different penetration enhancement mechanisms. Specifically, borneol is thought to cause opening of intercellular tight junctions (Kaur and Smitha, 2002; Kaur et al., 2004). Menthol may use the ability of its alcoholic hydroxyl group to disrupt the lipid hydrogen-bond network in cell membranes (Amnuaikit et al., 2005) or act on collagens to increase the fluidity of the lipid layer (Li et al., 2001). Labrasol can change the solubility properties of certain drugs and increase drug solubility. It has also been reported to increase the fluidity of the lipid bilayer (Liu et al., 2009).

When used alone, each penetration-enhancer can only increase permeability up to the point allowed by its particular method of action. For this reason, we studied the effects of combinations of penetration enhancers not only at the previously established optimal concentration, but also at half of these concentrations. At both concentrations, the penetration-enhancing effects were improved by the addition of Labrasol. This suggests that the mechanisms of borneol, menthol, and Labrasol are not identical, and by combining these compounds a synergistic effect can be achieved that makes it possible to attain increased levels of drug penetration. Previously, Thomas et al. demonstrated a favorable effect by combining menthol and eucalyptol to promote the penetration of Zidovudine (Thomas and Panchagnula, 2003). Our research group combined borneol and menthol in a previous study and found that the combination of the two significantly increased the corneal permeability of fluconazole (Liu et al., 2012). In this study we found that the combination of 0.1% borneol and 2% Labrasol produced an excellent effect, increasing \( P_{\text{app}} \) values by 15.35 times higher than that of the control group (table 4). The combination of 0.2% menthol and 2% Labrasol also increased \( P_{\text{app}} \) values by 13.43 times. Labrasol may produce this effect by improving the dissolution state of baicalin and increasing the fluidity of the lipid bilayer, acting synergistically to enhance the effects of menthol or borneol.

The current trend in drug application is the combined use of low-dose drugs. This approach can maximize desired effects while minimizing undesirable side-effects. Most drugs used in traditional Chinese medicine are extracted from well-known plants, and as such, their effects have

<table>
<thead>
<tr>
<th>Table 1: Penetration experiment groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
</tr>
<tr>
<td>No treatment</td>
</tr>
<tr>
<td>1% Labrasol</td>
</tr>
<tr>
<td>2% Labrasol</td>
</tr>
</tbody>
</table>

\*“a” represents the control group with no treatment.
\*“b” represents the single penetration enhancer group treated with different concentration of Labrasol, borneol and menthol as a single penetration enhancer.
\*“c” represents the compound penetration enhancer group treated with a combination of Labrasol with borneol or menthol as a new compound penetration enhancer.

<table>
<thead>
<tr>
<th>Table 2: Experimental grouping of the rabbits Draize test and components of testing drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
</tr>
<tr>
<td>Baicalin (mg/L)</td>
</tr>
<tr>
<td>200</td>
</tr>
</tbody>
</table>
Combined use of borneol or menthol with labrasol promotes penetration of baicalin

Table 3: The maximum safe concentrations and optimal penetration enhancing concentrations of three penetration enhancers

<table>
<thead>
<tr>
<th>Penetration enhancer</th>
<th>Maximum safe concentrations</th>
<th>Optimal penetration enhancing concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labrasol</td>
<td>3%</td>
<td>2%</td>
</tr>
<tr>
<td>Menthol</td>
<td>0.4%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Borneol</td>
<td>0.2%</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

Table 4: Regression formulas, apparent corneal permeability coefficient, and enhancement ratios of different types of penetration enhancers in rabbit cornea

<table>
<thead>
<tr>
<th>Penetration enhancer</th>
<th>Regression formula$^A$</th>
<th>$P_{app} \times 10^6$(cm/s)</th>
<th>ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Labrasol</td>
<td>$Q = 0.4566t – 0.1092$</td>
<td>$1.22 \pm 0.02$</td>
<td>1</td>
</tr>
<tr>
<td>1%</td>
<td>$Q = 0.9570t – 0.1525$</td>
<td>$2.59 \pm 0.01^a$</td>
<td>2.12</td>
</tr>
<tr>
<td>2%*</td>
<td>$Q = 2.0721t – 0.6995$</td>
<td>$5.47 \pm 0.12^a$</td>
<td>4.48</td>
</tr>
<tr>
<td>Menthol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1%</td>
<td>$Q = 1.5953t – 0.6509$</td>
<td>$4.16 \pm 0.06^a$</td>
<td>3.41</td>
</tr>
<tr>
<td>0.2%*</td>
<td>$Q = 2.2551t – 0.7542$</td>
<td>$5.95 \pm 0.19^a$</td>
<td>4.87</td>
</tr>
<tr>
<td>Borneol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05%</td>
<td>$Q = 1.7907t – 0.5933$</td>
<td>$4.73 \pm 0.08^a$</td>
<td>3.88</td>
</tr>
<tr>
<td>0.1%*</td>
<td>$Q = 2.4544t – 0.7565$</td>
<td>$6.50 \pm 0.12^a$</td>
<td>5.33</td>
</tr>
<tr>
<td>Menthol + Labrasol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1% + 1%</td>
<td>$Q = 2.3362t – 0.7982$</td>
<td>$6.16 \pm 0.10_{a,b,d}$</td>
<td>5.05</td>
</tr>
<tr>
<td>0.2%* + 1%</td>
<td>$Q = 3.2129t – 1.1240$</td>
<td>$8.46 \pm 0.23_{a,b,e}$</td>
<td>6.93</td>
</tr>
<tr>
<td>0.1% + 2%*</td>
<td>$Q = 4.7515t – 1.8267$</td>
<td>$12.49 \pm 0.21_{a,c,d}$</td>
<td>10.24</td>
</tr>
<tr>
<td>0.2%* + 2%*</td>
<td>$Q = 6.7695t – 2.8986$</td>
<td>$17.61 \pm 0.27_{a,c,e}$</td>
<td>14.43</td>
</tr>
<tr>
<td>Borneol + Labrasol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05% + 1%</td>
<td>$Q = 3.1675t – 0.9733$</td>
<td>$8.40 \pm 0.12_{a,b,f}$</td>
<td>6.89</td>
</tr>
<tr>
<td>0.1%* + 1%</td>
<td>$Q = 4.7915t – 1.7820$</td>
<td>$12.58 \pm 0.26_{a,b,g}$</td>
<td>10.31</td>
</tr>
<tr>
<td>0.05% + 2%*</td>
<td>$Q = 6.6633t – 2.6820$</td>
<td>$17.41 \pm 0.25_{a,c,f}$</td>
<td>14.27</td>
</tr>
<tr>
<td>0.1%* + 2%*</td>
<td>$Q = 7.6821t – 3.3855$</td>
<td>$19.95 \pm 0.20_{a,c,g}$</td>
<td>16.35</td>
</tr>
</tbody>
</table>

Acknowledgment

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